Cytogenetic Biodosimetry for Radiation Disasters: Recent Advances

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ABSTRACT

Potential scenarios of radiation exposure resulting in mass casualties require individual, early, and definitive diagnostic radiation dose assessment to help provide medical aid within days of the occurrence of a catastrophe. The long-range goal of the Armed Forces Radiobiology Research Institute (AFRRI) Biological Dosimetry Team is to identify, develop, and optimize broad dose-range biodosimetry methods, using in vitro models, and to validate those methods, using suitable in vivo models. Of these methods, the cytological biodosimetry methods are capable of assessing radiation dose both in whole-body and partial-body radiation exposure scenarios. Chromosome-aberration analysis in exposed individual's peripheral blood lymphocytes is an internationally accepted "gold standard" cytogenetic biodosimetry method that can be used to assess dose to help develop a treatment strategy within days of a radiation catastrophe. We have established the conventional lymphocyte metaphase-spread dicentric assay, in accordance with international harmonized protocols, and have used the assay to estimate radiation doses in several accidents. Current efforts focus on increasing sample throughput via automation. We systematically addressed the development of an automated cytogenetic laboratory that can triage by exposure group (not life-threatening, potentially life-threatening, and significantly life-threatening) and thereby efficiently differentiate radiation-exposed individuals from the "concerned public" following a disaster. Our studies included concept feasibility, workflow analysis, possible process reengineering, bottleneck elimination in manual processing, and proof-in-principle experiments. With automation, up to 500 samples per week can be analyzed in triage mode in which chromosome aberration analysis is restricted to 20 to 50 metaphase spreads per sample compared with the conventional approach of 500 to 1000 spreads. In another effort, we are developing and validating a novel cytogenetic bioassay, the rapid interphase chromosome aberration (RICA) assay, using suitable in vivo models. In this innovative method, radiation-induced chromosome aberrations in specific chromosomes are studied directly and rapidly in resting peripheral blood lymphocytes after inducing premature chromosome condensation either by fluorescence in situ hybridization or by immuno-enzymatic detection based on bright-field microscopy. The latter method is amenable to automation using image analysis. We used an accidentally radiation-exposed cohort, in Thailand in Feb. 2000, to determine the ability of the RICA assay to assess whole-body equivalent dose; our results indicated that the RICA assay can be used to assess radiation-induced damage to a specific chromosome in interphase cells. We are using a rodent model to determine the persistency of radiation-induced damage and the influence of sampling delay on dose estimation. These studies, in general, will contribute to an improved diagnostic response to a mass-casualty situation and will improve protection and survivability in adverse ionizing radiation environments.

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1.0 INTRODUCTION

Mettler [2002] reviewed potential radiation exposure scenarios which included detonation of nuclear weapons, terrorist attacks on nuclear reactors, and dispersal of radioactive substances with the use of conventional explosives, resulting in mass casualties. These disasters can result in different forms of radiation exposure: whole body, localized or partial body, internal contamination, external contamination, and contaminated burns and wounds. Strategies for triage and evacuation of the injured, contaminated, and non-contaminated casualties are proposed herein. The onset, nature, severity, and duration of clinical symptoms following radiation exposure are determined primarily by the casualty's absorbed dose but are also influenced by the radiation field and quality, the dose rate, and the individual's inherent radiosensitivity and general health status.

In reaction to prompt total-body ionizing radiation with a dose range of 0.5 to 30 Gy (photons), the typical symptoms of radiation exposure in humans include nausea, vomiting, diarrhea, and peripheral blood lymphocyte depletion [Anno 1989]. Some of these clinically significant radiation exposure symptoms are also seen in several other diseases. The duration of initial or prodromal symptoms and the latent phase of radiation syndrome is anywhere from 1 h to 2 weeks. Without appropriate medical care, the median lethal dose of radiation, the $LD_{50/60}$ (the dose that kills 50% of the exposed population within 60 days after exposure), is estimated to be 4.5 Gy [Mole 1984]. However, the likelihood of survival can be increased significantly with appropriate aggressive medical intervention and care [Anno 2003].

The practical pharmacological approaches to reducing the severity of radiation injury after a radiological emergency are limited by at least three factors [Moulder 2004]. First, most of the known pharmacological interventions are still experimental. Second, effective use of pharmacological intervention requires rapid and accurate determination of radiation dose. And third, in a large-scale public radiation disaster, medical facilities will be overwhelmed by large numbers of individuals who actually have not been exposed to radiation but fear they have been exposed. Practical methods of diagnostically separating a small number of radiation exposed individuals who require medical intervention from the large population of "concerned public" are critical. Triage following a radiation accident or a terrorist event should involve (i) a separation of victims with biologically relevant dose from the "concerned public" and (ii) a determination of priority of urgent care. Non-availability or inaccurate initial dose estimates, within hours to weeks after exposure, could result in suboptimal medical intervention. Therefore, for early treatment of radiation victims, it is recommended that medical personnel rely heavily on clinical signs and biological dose assessments [Goans 1997].

The workgroup established by the Office of Science and Technology Policy and the Homeland Security Council, to help the United States prepare for the possibility of a radiological disaster, deliberated on the research needs and identified and prioritized areas for attention. Automation of biodosimetry assays was identified as one of the six highest research priority areas [Pellmar 2005]. The objective of this recommended priority research area is to improve, through automation, the speed and efficiency of biodosimetric assays for triage and therapy. Despite the robustness and adaptability of existing biodosimetric approaches, the process is tedious and time-consuming with limited sample throughput. The present article addresses the applicability of cytogenetic assays in radiation mass casualties for confirmation of clinical triage and dose assessment, improved automated systems to increase sample throughput, and implementation of a Laboratory Information Management System (LIMS) to enhance good laboratory practice (GLP) for cytogenetic biodosimetry.

2.0 DICENTRIC ASSAY IN RADIATION MASS CASUALTIES

Cytogenetic biological dosimetry can make valuable contributions to the medical management of patients in the early period after a radiation disaster, where a rapid confirmation of dose is required. At such time, all that is needed is a rapid confirmation of clinical triage, based on approximate dose estimation using biological and clinical endpoints to a selected cohort, rather than precise dose estimations for a vast number of individuals. Radiation exposure induces many types of chromosomal aberrations in the exposed individual's peripheral blood lymphocytes. The presence of dicentrics, a chromosomal structural aberration, in an individual's peripheral blood lymphocytes indicates radiation exposure. Dicentrics are considered relatively radiation specific; only a few chemicals are known to interfere with the assay. Low background levels (about 1 dicentric in 2000 cells), high sensitivity (a threshold dose of 0.05 Gy), and known dose dependency of up to 5 Gy (for photons) make this assay robust and a "gold standard" biodosimetry method. This cytogenetic chromosome aberration bioassay is a thoroughly investigated biodosimetry method. The dicentric assay is conventionally used to provide definitive radiation dose assessment. Because exposure of human peripheral blood lymphocytes (HPBL) *in vitro* and *in vivo* produces similar levels of dicentrics per unit dose, dose estimation to an exposed individual can be made by comparing the observed frequencies of dicentrics with an *in vitro* generated dose-effect calibration curve [IAEA 2001].

The utility of cytogenetic assays to assess health risks and to guide medical treatment decisions was demonstrated in several radiation accidents involving mass casualties, such as those in Chernobyl, Goiania, and Tokaimura. Estimated doses using cytogenetic methods correlate well with the severity of acute radiation syndrome [Sevan'kaev 2000]. In the Chernobyl accident in Russia, an approximate dosimetry was achieved by rapid preliminary examination of 50 lymphocyte metaphases per person for several individuals [Pyatkin 1989]. Ramalho [1991] investigated 129 exposed or potentially exposed individuals from the Goiania, Brazil, accident cohort and dose estimates exceeded 1 Gy for 21 individuals and 4 Gy for 8 others. More recently, dose estimation was done using the dicentric and PCC assays in the Tokaimura, Japan, criticality accident in 3 severely exposed workers [Kanda 2002, Hayata 2001] and 43 resident workers [Sasaki 2001]. These radiation accidents highlight the importance of the cytogenetic methods in early dose assessment after a radiological event and demonstrate their ability to influence medical treatment decisions.

Recently, it was suggested that the dicentric assay could be adapted for the triage of mass casualties [Lloyd 2000, Voisin 2001, Prasanna 2003]. Lloyd [2000] described an *in vivo* simulation of an accident with mass casualties receiving whole- or partial-body irradiation in the 0- to 8-Gy range. Faced with an urgent need for rapid results, clinical triage was accomplished by scoring as low as 20 metaphase spreads per subject, compared with the typical 500 to 1000 spreads scored in routine analyses for estimating dose. However, Lloyd [2000] suggested increasing the analyses to 50 metaphase spreads when there is disagreement with the initial assessment or when there is evidence of significant inhomogeneous exposure. After the initial results are communicated to the treating physician, additional scoring is recommended to resolve potential conflicts in dose assessment and, in the case of high doses, to assist physicians considering bone marrow stem-cell transfusions to mitigate bone marrow ablation. Lloyd [1997], after studying lymphocyte chromosome damage in 10 of the 13 severely irradiated Chernobyl victims, suggested that the frequency of metaphase spreads without dicentric aberrations can be used to identify patients suitable for cytokine therapy versus bone-marrow transplantation. Early identification of radiation casualties, who will benefit from cytokine therapy, is relevant because current consensus medical management guidelines encourage an early administration of cytokines [Waselenko 2004]. Using the dicentric assay in the triage mode, a reasonable throughput of 500 or more samples per week per laboratory is achievable [Prasanna 2003]. However, a reach-back reference cytogenetic laboratory is required for assessing a rapid confirmation of radiation dose to individuals following a radiation disaster.

In a radiological mass-casualty incident, one or more of the following guidelines may be used to determine the cohort, which requires triage cytogenetic analysis in a reach-back reference cytogenetic biodosimetry laboratory.

- Time of emesis is less than 2 hours after radiation exposure
- An approximate 50% decline in peripheral blood lymphocyte counts over 12 hours
- Geographical location-based physical dosimetry indicating above 3-Gy dose
- Multi-parameter clinical symptoms indicative of an onset of acute radiation syndrome.

2.1 Reference Cytogenetic Biodosimetry Laboratory

AFRRI supports the U.S. Department of Defense's medical readiness by providing a limited cytogenetic biodosimetry service capability for radiation dose assessment conforming to international guidelines following the establishment of a "reach-back" cytogenetic biodosimetry laboratory [Prasanna 2002]. Blood samples (10 to 15 ml) are collected from the exposed individuals as soon as practical, generally one day after exposure, and are transported to the laboratory where lymphocytes are isolated from whole blood and stimulated to grow in culture, metaphase spreads are harvested, and chromosome aberration analyses are performed using internationally accepted laboratory protocols [IAEA 2001].

The blood collection procedure for cytogenetic biodosimetry is described in AFRRI's *Medical Management of Radiological Casualties Handbook* [AFRRI 2003]. This medical management doctrine can be downloaded from AFRRI's website, www.afrri.usuhs.mil. Since 2000, AFRRI's cytogenetic laboratory has analyzed more than 15 cases from radiation incidents and accidents, using dose-response calibration curves in HPBL [Prasanna 2002a] by dicentric assay, and 12 cases from a radiological accident that occurred in Thailand in February 2002 using the Rapid Interphase Chromosome Aberration (RICA) assay.

2.2 International Efforts on Cytogenetic Biodosimetry Method Harmonization

The biodosimetry scientific community realized the need to harmonize the cytogenetic dosimetric methodology because there is no universally adopted laboratory protocol and important variations occur between the laboratories, often influencing the outcome of results. AFRRI scientists are involved in these international efforts to harmonize the biodosimetry cytogenetic methods to address this problem. The International Atomic Energy Agency (IAEA) published a technical manual, involving the efforts of AFRRI scientists, on cytogenetic biodosimetry that provides a harmonized methodology for various cytogenetic assays. This manual [IAEA 2001] provides information necessary for selecting and implementing, in a standardized manner, the appropriate cytogenetic method to ensure accurate dose assessments following an accidental exposure to ionizing radiation.

An International Organization for Standardization (ISO) working group, comprised of several scientists from more than 12 countries and an IAEA representative, was established to standardize biological dosimetry by cytogenetics. Under the auspices of the ISO, regulatory compliance and validation efforts are being made for the dicentric assay. The scope and structure of the working draft, ISO TC-85/SC-2, Radiation Protection—Performance Criteria for Service Laboratories Performing Biological Dosimetry, provides the guidelines for conducting the biological dosimetry by cytogenetics [Voisin 2002].

Current efforts of this ISO workgroup focus on developing the standard entitled, "Radiation Protection—Performance Criteria for Service Laboratories Performing Cytogenetic Triage for Assessment of Mass casualties in Radiological and Nuclear Emergencies." Several cytogenetic biodosimetry laboratories have protocols

for rapid dose assessment using cytogenetic methods that involves pre-planning, reagent stockpiling, simplified and automated sample processing, modifying some of the ISO 19238 chromosome aberration analysis criteria, and networking with other expert laboratories for rapid dose assessment. The purpose of this new standard is to define the process and apply quality-control standards to components for triaging radiation mass casualties using cytogenetic methods. This standard will provide guidance on using cytogenetic methods to rapidly assess radiation doses received by individuals and supplement the early clinical categorization of casualties. This standard will specify adaptations required in the ISO 19238 document for applying cytogenetic analysis for radiation mass casualties, and define accreditation criteria for performing quality-assured cytogenetic triage. The "reach-back" cytogenetic biodosimetry laboratory in AFRRI is implementing laboratory procedures for good quality control and quality assurance.

3.0 PREMATURE CHROMOSOME CONDENSATION ASSAY

Conventional metaphase-spread chromosome-aberration biodosimetry techniques are robust, but they are laborious, time-consuming, and, more importantly, require an *in vitro* stimulation of resting HPBL to cause proliferation. For potential high-dose irradiation above the median lethal dose, such as in a radiation disaster, it is expected that radiation-induced cell death and delay in cell cycle progression into mitosis will interfere with dose estimation [Prasanna 2002b]. In addition, high-dose radiation accident victims will also suffer from lymphopenia; therefore, few cells will be available for cytogenetic studies. In order to overcome this limitation, quantitative analysis of radiation-induced damage may be performed using resting HPBL *in lieu* of metaphase spreads. Use of interphase cytological assays, such as the premature chromosome condensation (PCC) assay, could eliminate these inherent problems associated with the use of metaphase-spread cytogenetic assays. The PCC assay is useful to determine exposure to low doses as well as to life-threatening acute high doses of low-LET (linear energy transfer) [Prasanna 1997, 2000] and high-LET radiation [Prasanna 1997]. Moreover, the PCC assay can discriminate between total- and partial-body exposures [Blakely 1995].

3.1 Rapid Interphase Chromosome Aberration (RICA) Assay

It was shown that PCC can be induced in resting HPBL through signal transduction mechanisms by a simple incubation of cells in a culture medium containing a protein phosphatase inhibitor, okadaic acid, a mitosis-promoting factor $p34^{cdc2}$/cyclin B kinase [Prasanna 2000, 2005]. The interphase-based rapid interphase chromosome aberration (RICA) assay is a simple alternative to the metaphase-spread based dicentric assay. In the RICA assay, damage involving specific chromosomes is analyzed in chemically induced PCC spreads after fluorescence *in situ* hybridization (FISH) with specific whole-chromosome DNA hybridization probes (Prasanna and Blakely, international patent pending). In the RICA assay, the cells that display two chromosome spots are considered normal and cells with more than two chromosome spots are considered aberrant. The frequency of aberrant cells [Prasanna 2000] and the number of aberrations per cell [Prasanna 2002b] are shown to increase with radiation dose over a broad dose range encompassing those well above the median lethal dose. This cytogenetic assay has wide applications across biotechnology and biomedical fields. Our laboratory is now focussing on validating this rapid and simple dose assessment method using several *in vivo* irradiation models.

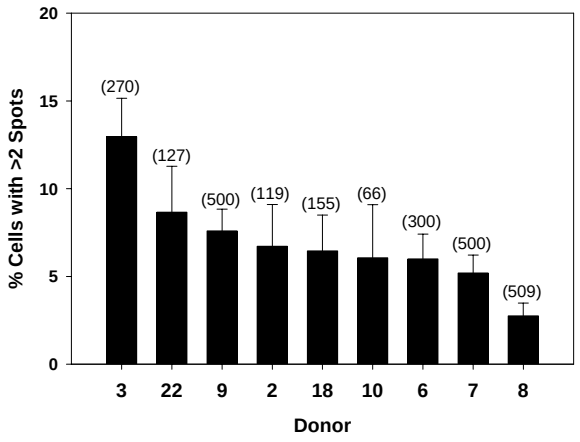
3.2 *In Vivo* Validation of the RICA Assay

3.2.1. Determination of Equivalent Whole-Body Dose to Radiation Exposed Individuals: Radiation Accident Cohort Model

Accidentally radiation-exposed human population cohorts provide a unique opportunity to test the utility of

newly developed biodosimetry methods for estimating health risks for individuals and population cohorts. These applied studies often guide clinical treatment decisions to the accidentally exposed human subjects. In February 2000, in Samutprakaran, on the outskirts of Bangkok, Thailand, several personnel were accidentally exposed to ^{60}Co gamma rays radiating from a scrap metal unshielded source in a recycling yard. The details of this radiological accident were published earlier [Jinaratana 2002, IAEA 2002]. The exposed personnel received acute, chronic, or fractionated exposures. The specific objective of this study was to demonstrate the utility of newly developed bioassays to determine equivalent whole-body dose to individuals. In a collaborative study, we compared the data obtained using the RICA assay with initial physical dosimetry and diagnostic white blood cell counts in a few individuals. The RICA assay was conducted as previously described [Prasanna 2000, 2004] 3 months post-exposure. Informed consent from all the exposed individuals and donors was obtained by the Department of Medical Science, National Institute of Health, Ministry of Public Health, Thailand, before blood collection for cytogenetic biodosimetry.

Figure 1: Chromosome 1 aberrations in individuals accidentally exposed to radiation in Thailand



The baseline frequency for the RICA assay in our laboratory is 0.006 ± 0.002. Figure 1 shows the frequencies of cells with aberrant chromosome 1 detected by the RICA assay in various donors. The number of cells analyzed is given in parentheses for each of the donors. Cells that showed more than two spots for chromosome 1 following PCC induction and fluorescence *in situ* hybridization (FISH) were considered aberrant. Cells with aberrant chromosome 1 ranged from 2.75 ± 0.73 in donor 8 to 12.96 ± 2.04 per 100 cells in donor 3. The number of aberrant cells in the un-irradiated control was 0.006 ± 0.002. Donor 2, 3, and 7 showed clinical symptoms of radiation sickness. Donor 3 worked in the scrap yard and as a maid for the scrap yard owner and the spouse. This patient was admitted to the hospital following complaints of nausea, vomiting, decreased appetite, and severe headache. Hair loss and mild fever also were noted following admission. A bone aspiration biopsy revealed a severe aplasia. The patient also showed a severe depletion in white blood cell count, which eventually was restored to normal after about 4–5 weeks. In donor 3, of the 270 cells analyzed, 12.96 ± 2.04 percent of the cells were aberrant. In donor 2, the scrap yard owner's 75-year-old mother, 6.72 ± 2.30 percent of the cells showed chromosome 1 aberrations. She also had a low white-blood cell count and complained of nausea, vomiting, and weakness with slight fever. The preliminary metaphase-spread chromosomal analysis, performed weeks after exposure, showed complex chromosomal changes, with the presence of dicentrics and ring chromosomes in some cells [IAEA 2002]. In donor 7, 5.2 ± 0.99 percent of the cells were aberrant. This individual also showed burns on fingers and complained of mild nausea, vomiting, and skin necrosis. Donors 6, 8, and 9 were temporary scrap yard workers, and 10 was a scrap yard office employee. These individuals apparently did not manifest clinical symptoms indicating radiation sickness but showed an increase in the frequency of cells with an aberrant chromosome 1. In this group, the frequency of aberrant cells ranged between 2.75 ± 0.73 and 7.6 ± 1.19 percent. Donors 18 and 22 lived within 50 to 100 meters from the scrap yard and frequented the site, but these individuals showed no signs or symptoms of radiation sickness despite a frequency of 6.45 ± 1.97 and 8.66 ± 2.50 percent of aberrant cells, respectively. The highest frequency of aberrant cells appeared in donor 3, who also displayed ARS symptoms such as nausea, vomiting, headache, epilation, and depleted white-blood-cell counts (Figure 1). Similarly, donor 7, who also manifested symptoms

of radiation sickness, showed 5.2 ± 0.99 percent aberrant cells in the PCC-FISH assay. This patient also showed a relatively higher frequency of aberrant cells using the FISH assay. A similar trend also was seen in temporary scrap yard workers (donors 8 and 9) and an office employee (donor 10). However, one of the nearby residents (donor 22) showed a relatively higher level of damage using the PCC FISH assay (8.66 ± 2.50 percent).

Figure 2: Correlation with *in vitro* dose response data for the RICA assay in Thailand radiation accident cohort

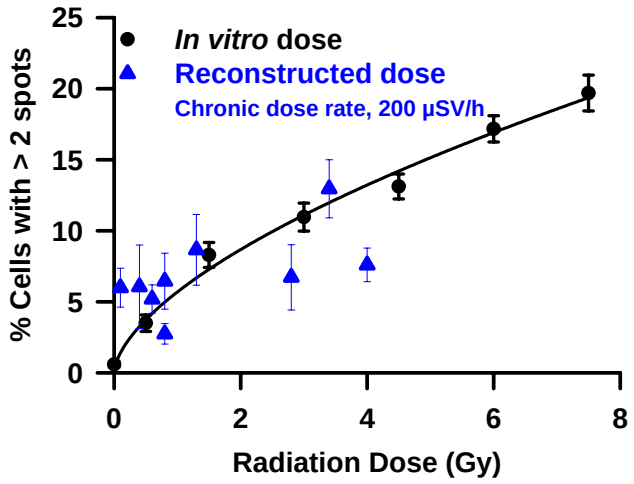


Figure 2 illustrates a correlation between percent aberrant cells showing chromosome 1 aberrations and reconstructed physical dose estimates for each of these individuals. Circles represent dose *vs.* percent aberrant cells (cells with more than 2 spots) as visualized by the RICA assay, plotted as a function of *in vitro* radiation dose [Prasanna 2000]. Triangles represent the percent of cells with more than 2 spots plotted against reconstructed physical dose. These results demonstrate the practical utility of the RICA assay for biological estimation of radiation dose in radiation accidents.

In this study, the assay was conducted after a sampling delay of 3 months after radiation exposure. At this post-exposure time-point, predominantly radiation induced symmetrical exchanges, akin to translocations and some residual asymmetrical exchanges (unstable aberrations such as dicentrics and fragments) may be observable because cells that carry unstable aberrations are mostly eliminated from the peripheral blood lymphocyte population. Therefore, it is imperative to study the persistency of the radiation-induced chromosome damage as visualized by the RICA assay.

3.2.2 Persistency of Radiation-Induced Chromosome Damage in the RICA Assay: Mouse Model

In general, radiation-induced "unstable" cytogenetic aberrations related to lymphocyte life-cycle decrease (e.g., dicentrics) over time after irradiation, particularly in peripheral blood lymphocytes correlating with lymphocyte lifespan. However, symmetrical translocation or "stable" aberrations persist over time. While metaphase spread-based dicentric and translocation assays have been characterized for persistency, the effect of sampling delay on persistency of damage needs to be characterized for the RICA assay.

Our laboratory addressed, in a proof-of-principle experiment, the persistency of radiation-induced cytogenetic damage for the RICA assay. Thirty days after a sublethal dose of gamma-irradiation, the RICA assay was conducted in a mouse model. The CD2F1 mice obtained from Harlan Laboratories (Indianapolis, IN, USA) were 8-Gy irradiated with a ^{60}Co gamma source at a dose rate of 0.6 Gy/min. Median lethal dose ($LD_{50/30}$) for this strain of mouse is 8.68 Gy [Landauer 2003]. The mice were euthanized, and blood collected by cardiac puncture for PCC analysis (modified for small sample volumes) [Prasanna 2000, 2004]. A chromosome-2 DNA hybridization probe was obtained from commercial suppliers (Cambio, Cambridge, UK) and the manufacturer's recommended protocol was used for *in situ* hybridization. Chromosome-2 probe was detected on PCC spreads on slides using a modified immuno-enzymatic bright-field microscopy RICA assay. Our laboratory originally described [Kolanko 2000] *in situ* detection of human pan-centromere DNA hybridization probe

of peripheral lymphocyte metaphase spreads using an immuno-enzymatic bright-field microscopy method. Table 1 shows cytogenetic damage as seen by the modified immuno-enzymatic bright-field microscopy RICA assay in CD2F1 mice following 8.0-Gy irradiation with ^{60}Co gamma-rays after 30 days. Cells that showed more than two chromosome-2 spots were considered aberrant. The number of cells that displayed more than 2 spots increased significantly with irradiation. A significant elevation ($p < 0.001$) in the frequency of aberrant cells could still be seen 30 days after irradiation. Similarly, a relative increase in the number of chromosome spots per cell was also increased significantly in irradiated mice ($p < 0.001$).

Table 1: Cytogenetic damage as seen by the modified immuno-enzymatic bright-field microscopy RICA assay in CD2F1 mice following 8.0-Gy irradiation with ^{60}Co gamma-rays after 30 days

		Number of cells with spots					
Treatment	Total	2	3	4	5	Percent aberrant cells ± SE	No. of spots/cell ± SE
Control	541	515	21	5	–	4.81 ± 0.92	2.06 ± 0.012
Irradiated	560	447	76	34	3	20.17 ± 1.20	2.27 ± 0.025

Time-related information on decay of cytogenetic damage is important in estimating radiation dose after a significant sampling delay for the RICA assay following a radiation accident as in the above Thailand radiation accident. In addition, determination of persistency of damage using the RICA assay using a mouse model is necessary to acquire sufficient knowledge to extrapolate the results to humans and recommend to the military a suitable mathematical model for dose assessment after a significant sampling delay following accidental acute exposure of troops.

4.0 LABORATORY AUTOMATION

We recommend the use of commercially available off-the-shelf instruments for laboratory automation for triaging radiation mass casualties for a rapid turnover of results. The throughput of the cytogenetic laboratory can be increased by using instruments such as robotic devices for handling blood and for isolating lymphocytes; microprocessor-controlled multi-pipettes for transferring reagents; automated metaphase harvesters and spreaders with modules for 20 to 50 slides for simultaneous staining; and automated instruments for DNA hybridization of centromere/whole chromosome-specific probes and immuno-enzymatic chromosome painting. These methods also will ensure quality control and quality assurance under the Good Laboratory Practice environment for conducting assays.

4.1 Automated Liquid Handling and High-Throughput Lymphocyte Isolation

As early as the late 1980s, the commercial sector had developed liquid-handling robots to dispend, dilute, and aspirate blood samples [Freidman 1986]. These systems are precise, accurate, and do not contaminate between specimens [Hanson 2001]. Use of automated liquid-handling robots for high-throughput, sterile isolation of lymphocytes from peripheral whole blood can eliminate a bottleneck in sample processing for cytogenetic analysis. We are now configuring and customizing an automated robotic system consisting of a high-throughput liquid handler, the TECAN Freedom EVO 200 (Tecan US, Research Triangle Park, NC, USA), for isolating lymphocytes from 1 to 100 whole-blood samples per run in less than three hours with minimal or no user interaction. This will provide a 20-fold increase in sample throughput compared with the manual procedure. Components of this liquid handler include a work deck for robotic liquid sample processing with a liq-

uid-handling arm, a pick-and-place arm, and a robotic manipulator with extended Z-axis. The work deck is customized to include centrifuge racks and carriers. These components and processes are controlled by specialized software. The robot is integrated with a swinging bucket-automated centrifuge enclosed in an engineered Biosafety Level 2 (See http://bmbl.od.nih.gov/sect3bs12.htm) environment to ensure sterility of the samples and the occupational safety of laboratory personnel. The automated centrifuge integrates with the work deck of the liquid handler and is designed for automatic sample processing without user interaction, allowing the loading and unloading of the centrifuge with a robotic arm. The stainless steel chamber of the centrifuge allows easy cleaning of blood in the event of a spill. The entire robotic lymphocyte isolation system provides a chain-of-custody of sample identification via bar coding of samples and destination tubes using a bar-code scanner for positive identification of horizontal and vertical bar codes at any position on the deck.

4.2 Metaphase Harvester and Spreader

A commercially available metaphase-spread harvesting device can eliminate the labor-intensive process of harvesting cells from short-term peripheral blood lymphocyte cultures. The system performs tasks such as centrifugation of cell suspensions, aspiration and safe disposal of supernatant, treatment with pre-warmed hypotonic solution, and automatic fixation of cells with an acetic acid: methanol fixative with minimal or no user interaction. A metaphase spreader will increase the throughput of the chromosome spread preparation process from cell suspension onto glass slides. This instrument provides the ability to control the chamber's temperature and humidity, which is known to influence the quality of metaphase spreads on microscope slides [Deng 2003].

4.3 Autostainer and Coverslipper

An autostainer provides a rapid and consistent method of staining slides with minimal human involvement. A minimum capability of staining 1000 slides is essential in dealing with a radiation mass-casualty situation. The stainer should allow full integration with an automatic coverslipper. Equipment that provides a quick and efficient method of applying coverslips on cytogenetic slides without human interference is essential to increasing throughput.

4.4 Metaphase Finder and Satellite Scoring Stations

Laboratory sample throughput can be increased significantly by using an automated metaphase finder [Weber 1992]. Therefore, specialized cytogenetic laboratories usually rely on automated metaphase-finder systems for locating suitable chromosome spreads for analysis [Lloyd 1990; Blakely 1995; Prasanna 2002a, 2003]. AFRRI's high-throughput metaphase-finding system automatically locates metaphase spreads, obtained from human peripheral blood lymphocyte cultures, on microscope slides under a bright-field microscope and relocates metaphase spreads for user review and aberration analysis. This system consists of a Pentium-processor computer (3.2 GHz), a camera with FireWire external bus (see http://developer.apple.com/firewire/), a high-quality microscope, an automated stage with auto-focus, and slide vacuum delivery robotics. The computer is loaded with automated metaphase-finding software and interactive automated scoring and annotation software for cytogenetic biodosimetry applications. The system's throughput is further enhanced by aberration analysis in multiple satellite locations by several users [Prasanna 2004].

5.0 LABORATORY INFORMATION-MANAGEMENT SYSTEM

We are developing and customizing a commercially available off-the-shelf LIMS (laboratory information management system) to track samples, use resources efficiently, and control and assure quality. Our fully de-

veloped LIMS, CytoTrack™, includes commercially available hardware (wireless bar-code readers, label printers, etc.), software components, and various customized modules for cytogenetic biodosimetry application. CytoTrack™ aids sequential and repetitive recording/sample tracking tasks performed at defined intervals. It helps to keep chain-of-custody for samples, allows sample prioritization and reprioritization (as often as required in sample processing after an accident), enables sample scheduling and status tracking, electronic data transfer between stations through a private intra-laboratory network, and facilitates system integration. This enhances sample throughput under Good Laboratory Practice (GLP) conditions.

Figure 3: Sample conditions screen module of LIMS

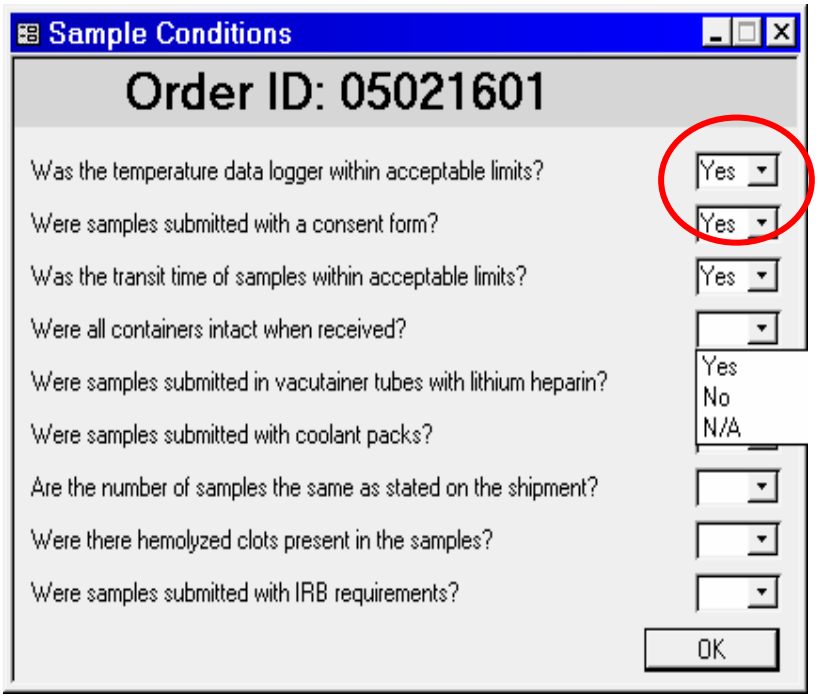
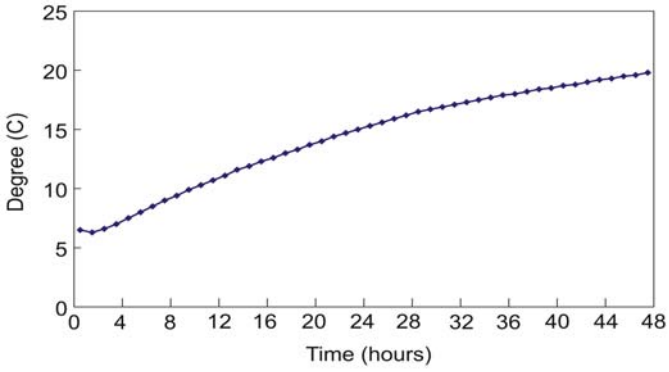


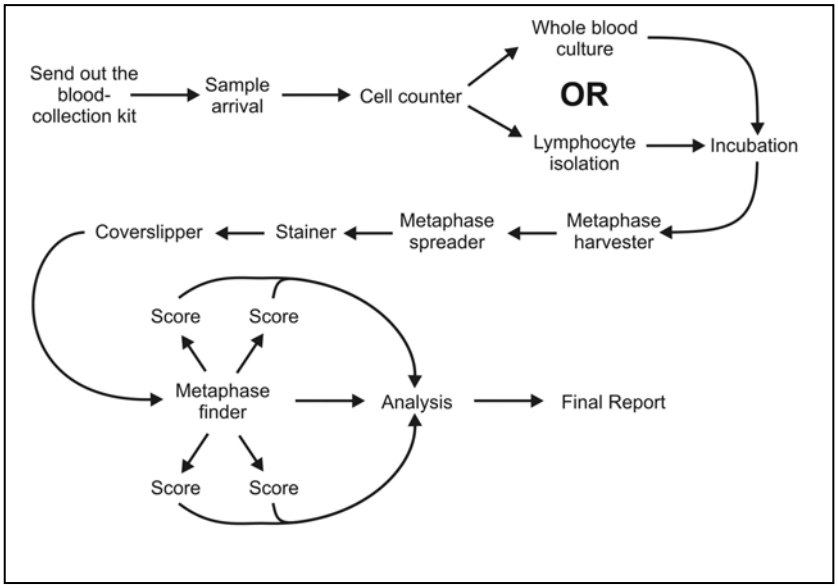
Figure 3 shows a sample module of this LIMS. The Sample Conditions module provides structured templates to input data on arrival conditions including temperature variation during transport. To determine sample integrity, a small, cost-effective, commercially available, temperature-data logger can be used to determine temperature changes during blood shipment from the collection site to the "reach back" cytogenetic laboratory. Temperature changes were measured using a pocket-sized data logger inside AFRRI's blood sample shipment kit for 48 hours, which contained freeze packs to keep the samples cold but not frozen during shipment, simulating a sample shipment. Temperature gradually rose with time from 5°C and reached close to room temperature (20°C) in about 2 days (Figure 4). These results are consistent with our previous observations that the samples can still used for cytogenetic assays after a 1-2 day transport under our defined conditions. For quality assurance, data from the above battery-operated data logger can be imported to the LIMS. Figure 5 shows the methodology used in the biodosimetry cytogenetic laboratory. While some of these time-consuming and rate-limiting processes are automated and require no user interaction (e.g., automated liquid handling), others require human intervention (e.g., automated metaphase-spread location and chromosome aberration analysis). This necessitates customization of equipment, the LIMS, system integration, and transition of manual protocols to an automated laboratory environment for increasing sample throughput. This will help achieve our cytogenetic biodosimetry technology transition objective of improving, through automation, the speed and efficiency of

Figure 4: Temperature changes measured via a pocket-size data logger inside AFRRI's blood shipment kit



the biodosimetric assays for confirmation of clinical triage and therapy.

Figure 5: Illustration of cytogenetic biodosimetry methodology



6.0 SUMMARY

AFRRI has addressed the following tasks:

- Establish a “reach-back” cytogenetic biodosimetry reference laboratory that uses dicentric and premature chromosome condensation assays to confirm individual radiation dose exposure.
- Develop an automated high-throughput cytogenetic laboratory to help triage radiation-exposed individuals into three categories: “not life-threatening,” “potentially life-threatening,” and “significantly life-threatening.”
- Develop and customize a commercially available off-the-shelf LIMS to track samples, efficient use of resources, quality control, and quality assurance.
- Support ISO’s efforts to draft a standard titled, “Radiation Protection—Performance Criteria for Service Laboratories Performing Cytogenetic Triage for Assessment of Mass Casualties in Radiological and Nuclear Emergencies.”

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8.0 REFERENCES

[AFRRI 2003] Medical Management of Radiological Casualties, Second Edition. Armed Forces Radiobiology Research Institute, Bethesda, MD.

[Anno 1989] G.H. Anno, S.J. Baum, H.R. Withers, R.W. Young, Symptomatology of acute radiation effects in humans after exposure to doses of 0.5–30 Gy. Health Physics 56: 821-838.

[Anno 2003] G.H. Anno, R.W. Young, R.M. Bloom, J.R. Mercier, Dose response relationships for acute ionizing-radiation lethality. Health Physics 84: 565-575.

[Blakely 1995] W.F. Blakely, P.G.S. Prasanna, C.J. Kolanko, M.D. Pyle, D.M. Mosbrook, A.S. Loats, T.L. Rippeon, H. Loats, Application of the premature chromosome condensation assay in simulated partial-body radiation exposures: Evaluation of the use of an automated metaphase finder. Stem Cells 13 (Suppl. 1): 223-230.

[Deng 2003] W. Deng, S.W. Tsao, J.N. Lucas, C.S. Leung, A.L. Cheung, A new method for improving metaphase chromosome spreading. Cytometry 51: 46-51.

[Friedman 1986] L.I. Friedman, M.L. Severns, Application of robotics in blood banking. Vox Sang (Suppl. 1): 57-62.

[Goans 1997] R.E. Goans, E.C. Holloway, M.E. Berger, R.E. Ricks, Early dose assessment following severe radiation accidents. Health Physics 72: 513-518.

[Hanson 2001] K.L. Hanson, C.P. Cartwright, Evaluation of an automated liquid-handling system (Tecan Genesis RSP 100 in the Abbott LCx assay for *Chlamydia trachomatis).* Journal of Clinical Microbiology 39: 1975-1977.

[Hayata 2001] I. Hayata, R. Kanda, M. Minamihisamatsu, M. Furukawa, M.S. Sasaki, Cytogenetic dose estimation for 3 severely exposed patients in the JCO criticality accident in Tokaimura. Journal of Radiation Research 42: S149-155.

[Hofmann 1990] R. Hoffman, G.A. Schreiber, N. Willich, R. Westhaus, K.W. Bogl, Increased serum amylase in patients after radiotherapy as a probable bioindicator for radiation exposure. Strahlentherapy Onkol., 166: 688-95.

[IAEA 2001] IAEA, Cytogenetic analysis for radiation dose assessment, a manual, Technical Report Series No. 405. International Atomic Energy Agency 2001, Vienna, Austria.

[IAEA 2002] IAEA, The radiological accident in Samut Prakaran. International Atomic Energy Agency, Vienna, 2002.

[Jinaratana 2002] V. Jinaratana, The radiological accident in Thailand. In: “Medical Basis of Accident Preparedness: The Clinical Care of Victims”, R.C. Ricks, M.E. Berger, F.M. O’Hara Jr. Parthenon Publishing, Boca Raton, FL, pp. 283-301.

[Kanda 2002] R. Kanda, M. Minamihisamatsu, I. Hayata, Dynamic analysis of chromosome aberrations in

three victims of the Tokaimura criticality accident. International Journal of Radiation Biology 78: 857-862.

[Kolanko 2000] C.J. Kolanko, M.D. Pyle, J. Nath, P.G.S. Prasanna, H. Loats, W.F. Blakely, *In situ* detection of a PCR-synthesized human pancentromeric DNA hybridization probe by color pigment immuno-staining: Application for dicentric assay automation. Biotechnique and Histochemistry 75: 91-98.

[Landauer 2003] M.R. Landauer, V. Srinivasan, T.M. Seed, Genistein treatment protects mice from ionizing radiation injury. Journal of Applied Toxicology 23: 379-385.

[Lloyd 1990] D.C. Lloyd, Advances in Cytogenetic Dosimetry, The Medical Basis of Radiation Accident Preparedness II: Clinical Experience and Follow-ups Since 1979, R.E. Ricks, S.S. Fry (Eds.), Elsevier, New York, NY, pp. 479-487.

[Lloyd 1997] D.C. Lloyd, Chromosomal analysis to assess radiation dose. Stem Cells (Supplement 2): 195-201.

[Lloyd 2000] D.C. Lloyd, A.A. Edwards, J.E. Moquet, Y.C. Guerrero-Carbajal, The role of cytogenetics in early triage of radiation casualties. Applied Radiation and Isotopes 52: 1107-1112.

[Mettler 2002] F.A. Mettler Jr., G.L. Voelz, Current concepts: Major radiation exposure—What to expect and how to respond. The New England Journal of Medicine 346: 1554-1561.

[Mole 1984] R.H. Mole, The LD50 for uniform low LET irradiation of man, The British Journal of Radiology 57: 355-369.

[Moulder 2004] J.E. Moulder, Post-irradiation approaches to treatment of radiation injuries in the context of radiological terrorism and radiation accidents: a review. International Journal of Radiation Biology 80: 3-10.

[Pellmar 2005] T.C. Pellmar, S. Rockwell, Priority research areas for radiological nuclear threat countermeasures. Radiation Research 163: 115-123.

[Prasanna 1997] P.G.S. Prasanna, C.J. Kolanko, H. M. Gerstenberg, W.F. Blakely, Premature chromosome condensation assay for biodosimetry: Studies with fission-neutrons, Health Physics 72: 594-600.

[Prasanna 2000] P.G.S. Prasanna, N.D. Escalada, W.F. Blakely, Induction of premature chromosome condensation by a phosphatase inhibitor and a protein kinase in unstimulated human peripheral blood lymphocytes: A simple and rapid technique to study chromosome aberrations. Mutation Research. 466: 131-41.

[Prasanna 2002a] P.G.S. Prasanna, H. Loats, H.M. Gerstenberg, B.N. Torres, C.W. Shehata, K.L. Duffy, R.S. Floura, A.W. Khusen, W.E. Jackson, W.F. Blakely, AFRRI's gamma-ray, x-ray, and fission-neutron calibration curves for the dicentric assay: Application of a metaphase finder system. AFRRI Special Publication 02-1, Defense Technical Information Center, Fort Belvoir, VA.

[Prasanna 2002b] P.G.S. Prasanna, C.J.C. Hamel, N.D. Escalada, K.L. Duffy, W.F. Blakely, Biological dosimetry using human interphase peripheral blood lymphocytes. Military Medicine, 167(Suppl. 1): 10-12.

[Prasanna 2003] P.G.S. Prasanna, U. Subramanian, R.G. Greenhill, J.M. Jacocks, W.E. Jackson, W.F. Blakely, Cytogenetic biodosimetry strategy for potential radiation mass casualties. In: "Procs. of the 36th Health Physics Society Midyear Topical Meeting, Radiation Safety Aspects of Homeland Security and Emer-

gency Response, San Antonio, TX, pp. 218-222.

[Prasanna 2005] P.G.S. Prasanna, W.F. Blakely. Premature chromosome condensation in human resting peripheral blood lymphocytes for chromosome aberration analysis using specific whole-chromosome DNA hybridization probes. Methods in Molecular Biology 291: 49-57

[Pyatkin 1989] E.K. Pyatkin, V.Y. Nugis, A.A. Chirkov, Absorbed dose estimation according to the results of cytogenetic investigations of lymphocyte cultures of persons who suffered in the accident at the Chernobyl atomic power station. Radiation Medicine: 4: 52.

[Ramalho 1991] A.T Ramalho, A.C. Nascimento, The fate of chromosomal aberrations in ^{137}Cs-exposed individuals in the Goiania radiation accident. Health Physics 60: 67-70.

[Sasaki 2001] M.S. Sasaki, I. Hayata, N. Kamada, N. Kodama, S. Kodama, Chromosome aberration analysis in persons exposed to low-level radiation from JCO criticality accident in Tokai-mura. Journal of Radiation Research: 42: S107-116.

[Sevankaev 2000] A.V. Sevankaev, Results of cytogenetic studies of the consequence of the Chernobyl accident. Radiation Biology Radioecology: 40: 589-595.

[Voisin 2001] P. Voisin, M. Benderitter, M. Claraz, V. Chambrette, I. Sorokine-Durm, M. Delbos, V. Durand, A. Leory, N. Paillole, The cytogenetic dosimetry of recent accidental overexposure. Cellular and Molecular Biology 47: 557-564.

[Voisin 2002] P. Voisin, F. Barquinero, W.F. Blakely, C. Lindholm, D. Lloyd, C. Luccioni, S. Miller, F. Palitti, P.G.S. Prasanna, G. Stephan, H. Thierens, I. Turai, D. Wilkinson, A. Wojcik, Towards a standardization of biological dosimetry by cytogenetics. Cellular and Molecular Biology 48: 501-504.

[Waselenko 2004] J.K. Waselenko, T.J. MacVittie, W.F. Blakely, N. Pesik, A.L. Wiley, W.E. Dickerson, H. Tsu, D.L. Confer, N.C. Coleman, T. Seed, P. Lowry, J.O. Armitage, N. Dainiak, Strategic National Stockpile Radiation Working Group, Medical management of the acute radiation syndrome: Recommendations of the Strategic National Stockpile Radiation Working Group. Annals of Internal Medicine 15:1037-1051.

[Weber 1992] J. Weber, W. Scheid, H. Traut, Time saving in biological dosimetry by using the automatic metaphase finder, Metafer2. Mutation Research 272: 31-34.